Investigations on Trypsin-Hydrolyzed Peptides for Protein Identification

A chromatographic method was developed for the identification of protein species through their peptide patterns. Protein isolates of beef, pork, chicken, and soy were heated, enzymatically hydrolyzed at optimal conditions, and subsequently analyzed by sequential application of thin-layer chromatography and high-performance liquid chromatography. The chromatographic patterns of the tryptic peptides were then statistically analyzed by discriminant analysis. A classification rule was derived to identify the proteins. Results showed that beef, pork, chicken, and soy proteins were significantly ($p < 0.05$) identified. Preliminary studies indicated that an all-beef frankfurter can be discriminated from a standard frankfurter containing 35% pork protein. Further studies are required to detect and measure the presence of "nonmeat" proteins as an adulterant or additive in processed meat products.

There is a continuing demand for development of a simple and rapid analytical method to detect, quantitate, and identify nonmeat proteins in processed meat products. Currently available methods have not been generally accepted. Recent reviews by Eldridge (1981), Eldridge and Wolf (1980), and Olsman and Krol (1978) describe the principles, advantages, and disadvantages of existing analytical techniques. Olsman (1979) categorized these techniques into two broad groups: (1) identification and analysis of substances accompanying nonmeat proteins by chemical analysis or histological and microscopic studies and (2) characterization of proteins through the analysis of their hydrolysis products (amino acids or peptides) and their physicochemical properties. Immunochemical techniques employed to detect the presence of protein additives were also cited in the preceding review papers.

The detection of nonmeat protein through its peptide hydrolysis products merits further investigation since it is simple and rapid. Furthermore, the peptide hydrolysates can be subjected to various analytical techniques. Bailey and co-workers (Bailey, 1976; Llewellyn et al., 1978; Bailey et al., 1978) measured the presence of soy protein in laboratory-fabricated soy–meat products by ion-exchange chromatographic analysis of trypsin hydrolysates of heat-denatured proteins. We have developed a modification of this method and investigated the identification of beef, pork, chicken, and soy proteins.

This paper presents a preliminary report on the sequential application of thin-layer chromatography (TLC) and high-performance liquid chromatography (HPLC) to peptide hydrolysates of selected proteins (beef, chicken, pork, and soy) previously heated and enzymatically hydrolyzed at optimal conditions. The chromatographic patterns of these peptides were statistically analyzed, and a classification rule was derived to identify the proteins.

EXPERIMENTAL SECTION

Preparation of Protein Isolates. Isolates of selected animal proteins were prepared by solvent extraction using acetone and ether (Morton, 1955). Fresh samples of beef, chicken, and pork (100 g each) were homogenized by blending with 200 mL of phosphate buffer (0.01 M, pH 6.5). The proteins were precipitated by adding 750 mL of cold acetone. The precipitate was allowed to settle for 30 min at 4 °C and the clear acetone layer was decanted. The precipitate was separated by centrifugation at 4080g, blended in 300 mL of 1-butanol, and then washed 2 times with 300 mL of petroleum ether to extract lipids. The acetone-precipitated proteins were dried under a stream of nitrogen and pulverized with a blender. The commercial soy isolate contained 92.5% protein and was utilized without further purification.

Preparation of Hydrolysates. The "superdenaturation" and hydrolysis of the proteins developed by Bailey (1976) were refined. Simultaneous optimization studies were employed on beef isolates to select the precise heating and hydrolysis conditions that would generate a fairly large number of resolvable fractions. The optimal heating temperature and time selected should be higher than those used in the processing of meat products.

Table I. Total Optical Density Ratings of Peptide Peak(s) in Nine HPLC Subfractions of Fraction IV

sample no.	sample type	trial	repli-cation	optical density ratings at elution time								
				3.4-3.6 $X_1{}^a$	4.7-5.1 X_2	5.2-5.6 X_3	5.7-6.3 X_4	6.8-7.2 $X_5{}^a$	8.9-9.8 X_6	12.0-12.4 $X_7{}^a$	12.8-13.2 X_8	13.5-14.8 $X_9{}^a$
1	beef	1	a	10	4	10	12	8	6	0	4	4
2	beef	1	b	10	0	8	8	8	6	4	0	4
3	beef	2	a	10	10	0	0	6	4	0	0	10
4	beef	2	b	10	10	0	4	6	4	0	6	4
5	soy	1	a	0	10	4	0	0	0	0	0	0
6	soy	1	b	0	10	4	0	0	0	0	4	0
7	soy	2	a	0	14	4	0	0	0	0	4	0
8	soy	2	b	0	10	4	0	0	0	0	4	0
9	chicken	1	a	10	10	4	8	6	6	10	0	10
10	chicken	1	b	10	0	12	8	6	4	10	0	10
11	chicken	2	a	10	18	0	0	6	4	0	10	10
12	chicken	2	b	6	8	6	8	6	6	10	0	10
13	pork	1	a	10	8	0	6	4	8	10	0	10
14	pork	1	b	10	4	8	4	6	4	10	0	10
15	pork	2	a	10	10	4	4	4	6	0	10	8
16	pork	2	b	10	0	6	4	4	4	6	10	0

a Significant ($p < 0.05$) variation of optical density ratings due to sample type.

The beef protein isolates (100 mg each) were hydrated with 1 mL of distilled water 30 min prior to autoclaving at 15 psi (121 °C) for 0, 30, 60, 120, and 180 min. The heated proteins were suspended in 3 mL of 0.05 M Tris–0.0086 M $CaCl_2$ buffer (pH 8.1) and hydrolyzed with 100 μL of 0.1% aqueous trypsin (chymotrypsin free) for 6, 12, 18, and 24 h at 24 and 37 °C. Acidification with 0.5 mL of glacial acetic acid stopped the hydrolysis and resulted in a pH of 3.2, which also precipitated the undigested proteins. Hydrolysates were centrifuged at 3020g and washed 2 times with 1.0% acetic acid. Aliquots of the supernatant were either taken for immediate analysis, stored frozen, or lyophilized for later analysis.

TLC Analysis. The protein hydrolysates containing approximately 10 μg of nitrogen/10 μL were applied to Kontes prelined silica gel TLC plates (5 × 20 cm; 250-nm thickness) treated with a preabsorbent for sample application. Plates were developed according to Brenner et al. (1969) in 2:2:1 chloroform–methanol–17% ammonium hydroxide, and the solvent was allowed to migrate 12 cm from the origin (ca. 90 min). The TLC plates were air-dried, and spots were visualized by spraying with 0.2% ninhydrin in acetone. The plates were again air-dried for 15 min and were heated at 85 °C for 5 min to develop the color. Semiquantitative data on the peptides complexed with ninhydrin were obtained by scanning with a Gelman ACD-18 densitometer at a wavelength of 525 nm.

Similar conditions were applied when TLC was used as a preparative step to separate the peptide hydrolysates prior to HPLC analysis. Development was carried out to a distance of 15 cm to achieve better separation of the peptide fractions. On each TLC plate, one channel was visualized with ninhydrin as a marker for the peptide separations.

HPLC Analysis. Tryptic hydrolysates separated by TLC were subdivided into four broad fractions (I, II, III, and IV) (Figure 1). Each TLC fraction was scraped off, extracted with 0.01% acetic acid, and centrifuged. Extracts were passed through a 45-μm filter, lyophilized, and reconstituted with microfiltered deionized water. Aliquots were taken for reverse-phase HPLC analysis at isocratic conditions using triethylamine–phosphate buffer (0.0833 M, pH 3.0) as a mobile phase at a flow rate of 1.0 mL/min (Rivier, 1978). The HPLC (Water Associates, Model 440) was equipped with C_{18} μBondapak column (3.9 mm i.d. × 30 cm) and a UV detector at a wavelength of 254 nm.
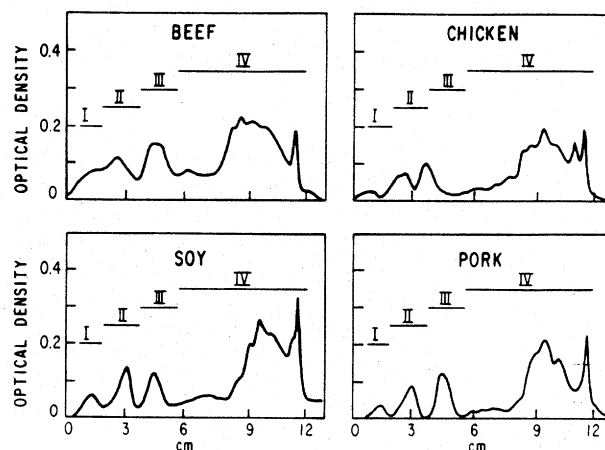

Figure 1. TLC densitometric scans at 525 nm of the peptide–ninhydrin complex of beef, soy, chicken, and pork subdivided into fractions I–IV.

Statistical Analysis. The HPLC separations of fraction IV were statistically evaluated since fractions I–III had identical peptide patterns in all proteins analyzed and therefore were not statistically different. The elution times (minutes) of the fraction IV peptides were subdivided into nine subfractions, and each was assigned an optical density rating corresponding to the magnitude of the peak(s) in that subfraction. The optical density of the subfractions (Table I) was rated from 0 to 10, assigning the following ratings to the optical density of each peak: 10 (≥ 0.041), 8 (0.031–0.04), 6 (0.021–0.03), 4 (0.011–0.02), and 0 (<0.01) (Figure 2). Individual ratings were added in subfractions containing more than one peak. These data were statistically analyzed by discriminant analysis (Rao, 1973) to determine if a classification rule could be derived for identification of protein samples. This analysis is based on the classification of an unknown protein sample into one of the classes of standard proteins through linear combinations of the optical densities of the protein subfractions. Analysis of variance was also performed to study the reproducibility of the experiments and to determine the sources of significant variability.

RESULTS AND DISCUSSION

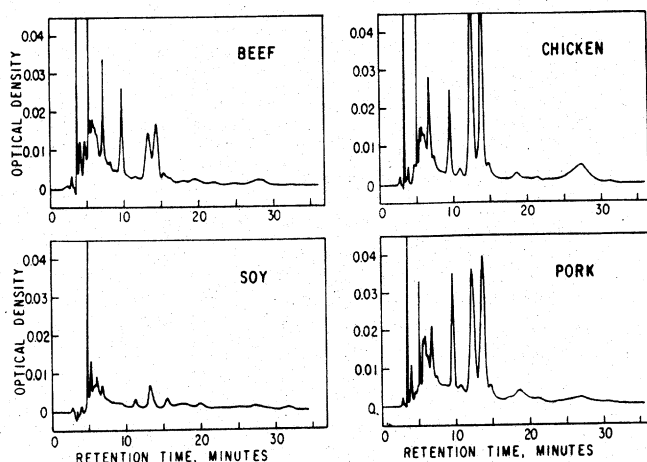Results of the combined optimization studies, as shown by TLC analysis, indicated that 60-min autoclaving at 15

Figure 2. HPLC peptide patterns of the fraction IV beef, soy, chicken, and pork.

Table II. Classification Scores for the Identification of Samples to a Particular Type of Protein

sample no.	type of protein used	computer-predicted type	classification scores			
			beef	chicken	pork	soy
1	beef	beef	107.8[a]	99.3	93.8	0
2	beef	beef	108.1[a]	102.1	97.0	0
3	beef	beef	90.2[a]	89.1	85.8	0
4	beef	beef	83.0[a]	78.9	79.2	0
5	soy	soy	−97.2	−85.1	−76	0[a]
6	soy	soy	−97.2	−85.1	−76	0[a]
7	soy	soy	−97.2	−85.1	−76	0[a]
8	soy	soy	−97.2	−85.1	−76	0[a]
9	chicken	chicken	91.0	96.1[a]	93.8	0
10	chicken	chicken	91.0	96.1[a]	93.8	0
11	chicken	beef[b]	90.2[b]	89.1	85.8	0
12	chicken	chicken	50.6	57.7[a]	51.0	0
13	pork	pork	66.2	75.7	79.2[a]	0
14	pork	chicken[b]	88.4	91.3[b]	90.0	0
15	pork	pork	63.0	65.3	69.0[a]	0
16	pork	pork	53.9	55.9	65.0[a]	0

[a] Highest scores indicating matching identity of sample and type of protein predicted. [b] Misclassified observation.

psi (121 °C) followed by tryptic hydrolysis was sufficient to produce a fairly large number of resolvable peptide fractions. The optical densities of these fractions were higher than those autoclaved at 120 and 180 min but lower than those from 0 and 30 min of autoclaving. Heating at 121 °C for 60 min was considered a minimal heat treatment to denature the proteins but was higher than commercial cooking conditions employed in the processing of meat products. Longer heat treatment, such as 120 and 180 min, could render the proteins resistant to enzymatic digestion due to chemical changes of the peptide linkages resulting in lower optical density of hydrolysates. Extensive heating of proteins also resulted in decreased chemical interaction of the peptide linkages indicated by reduction in copper complexation of the proteins (Medina, 1978).

The combination of optimum autoclaving time (60 min) and enzymatic hydrolysis conditions (18 h at 37 °C) was subsequently used to prepare the protein hydrolysates of beef, soy, chicken, and pork. The analysis of the tryptic digests by one-directional TLC showed highly reproducible separations, but resolution was poor, particularly among the less polar fractions. Attempts to further resolve these fractions by employing a second directional TLC analysis (Brenner et al., 1969), combinations of TLC and plate electrophoresis (Stephens, 1978), various buffer solutions, and developing solvents did not improve the results.

The less polar fractions in the TLC analysis were poorly resolved, but differences in peptide patterns among the proteins investigated were apparent (Figure 1). The TLC separations were grouped into four fractions, designating fraction I for the most polar peptides and fraction IV for least polar peptides. The subsequent HPLC analysis of these fractions indicated that elution times of the major peaks of fractions I–III were identical, varying only in optical density. The HPLC scans of the chicken and pork have similar patterns differing only in optical density and the ratio of the major peaks. While the more polar peptides of pork and chicken displayed a similarity to beef, the ratio of the less polar to more polar fractions was lower in the beef hydrolysates. Soy hydrolysates were characterized by the presence of only one major polar fraction and the absence of the less polar fractions found in the animal-derived proteins.

These characteristic peptide patterns are presumably influenced by two factors: (1) the dominance of nonpolar amino acids (methionine in actin and myosin and alanine in elastin) from animal-derived proteins and the dominance of the more polar amino acids (arginine, aspartic and glutamic acids) in soy protein and (2) relative amounts of

the specific amino acids for enzymatic cleavage. Trypsin specifically cleaves the carboxyl terminal of the lysyl and arginyl residues. Lysyl residue is lower in soy protein, and it is found mostly in the myosin and actin fractions of the animal-derived proteins. Therefore, trypsin hydrolysis of animal-derived proteins yields a peptide residue dominant in nonpolar amino acids. These assumptions were based on the amino acid content of proteins reported by FAO (1970) and Block and Weiss (1956).

Analysis of variance of the optical density ratings vs. elution time (Table I) showed that samples autoclaved, hydrolyzed, and chromatographed separately in two trials were not significantly different ($p < 0.05$). Results of duplicate analysis were not statistically different. However, the analysis of variance showed that subfractions X_1, X_5, X_7, and X_9 demonstrated a significant ($p < 0.05$) variation due to type of protein samples. The optical density ratings from these subfractions were subsequently used in the discriminant analysis, and a model for the classification rule was derived as

$$S_1(\text{beef}) = 10.1X_1 + 12.4X_5 + 0.08X_7 + 1.2X_9 - 97.2 \tag{1}$$

$$S_2(\text{chicken}) = 9.6X_1 + 10.2X_5 + 0.7X_7 + 1.7X_9 - 85.1 \tag{2}$$

$$S_3(\text{pork}) = 10.7X_1 + 7.3X_5 + 0.8X_7 + 1.1X_9 - 76.0 \tag{3}$$

$$S_4(\text{soy}) = 0 \tag{4}$$

By substitution of the X_i (optical density ratings) values of the individual protein samples into eq 1–4, the scores of each sample analyzed were calculated. The equation giving the highest score for a specific sample classified or identified that sample as the type of protein from which the equation was derived. For example, a beef protein was accurately identified when the beef equation (eq 1) gave the highest score. Results of this discriminant analysis showed that the samples were significantly ($p < 0.05$) classified as beef, pork, chicken, or soy protein. This classification rule identified 14 out of 16 samples (Table II) while samples no. 11 (chicken) and 14 (pork) were not correctly identified. These two discrepancies could be attributed to the variability of the HPLC elution, which in turn can be alleviated by a more thorough cleanup of the chromatographic column between samples or by using a mobile phase that can increase resolutions between peaks.

Heating conditions can change the type and number of peptide fractions resolved by TLC analysis. The classification rule derived from the sequential TLC–HPLC analysis of the tryptic hydrolysates demonstrated a high degree of accuracy in discriminating and identifying proteins analyzed individually. This chromatographic analysis and statistical approach could be employed to detect nonmeat protein as an adulterant or additive in processed meat products. When a product is claimed to be all beef, the beef equation (eq 1) should give the highest score; otherwise, the product can be suspected of adulteration or indiscriminate use of nontraditional "meat" in meat products. Preliminary results indicate that a laboratory-formulated all-beef frankfurter can be discriminated from a standard frankfurter containing 35% pork protein.

Quantitation of the adulterant or protein additive is possible by establishing a calibration standard of protein mixtures. This would require a different statistical approach from what has been employed for the qualitative detection of adulterants or additives.

LITERATURE CITED

Bailey, F. J. *J. Sci. Food Agric.* **1976**, *27*, 827–830.
Bailey, F. J.; Llewellyn, J. W.; Hitchcock, C. H. S.; Dean, A. C. *Chem. Ind. (London)* **1978**, *13*, 477–478.
Block, R. J.; Weiss, K. W. "Amino Acid Handbook"; Charles C. Thomas: Springfield, IL, 1956; pp 249–337.
Brenner, M.; Niederwieser, A.; Pataki, G. "Thin Layer Chromatography"; Stahl, E., Ed.; Springer-Verlag: New York, 1969; pp 730–786.
Eldridge, A. C. *J. Am. Oil Chem. Soc.* **1981**, *58*, 483–485.
Eldridge, A. C.; Wolf, W. J. *Proc.—World Soybean Res. Conf., 2nd, 1979* **1980**, 118–145.
FAO (Food and Agricultural Organization) "Amino Acid Content of Foods and Biological Data on Proteins"; Food and Agricultural Organization of the United Nations: Rome, 1970; pp 36–139.
Llewellyn, J. W.; Dean, A. C.; Sawyer, R.; Bailey, F. J.; Hitchcock, C. H. S. *J. Food Technol.* **1978**, *13*, 249–252.
Medina, M. B. Ph.D. Thesis, Rutgers University, 1978, pp 94–119.
Morton, R. K. *Methods Enzymol.* **1955**, *1*, 25–51.
Olsman, W. J. *J. Am. Oil Chem. Soc.* **1979**, *56*, 285–287.
Olsman, W. J.; Krol, B. "Methods for Detection and Determination of Vegetable Proteins in Meat Products", Report of the Study Group on Vegetable Proteins in Foodstuffs for Human Consumption in Particular in Meat Products EUR 6026 Commission of the European Communities; Luxemburg, 1978; pp 112–140.
Rao, C. R. "Linear Statistical Inference and Its Applications", 2nd ed.; Wiley: New York, 1973; pp 574–581.
Rivier, J. E. *J. Liq. Chromatogr.* **1978**, *1*, 343–366.
Stephens, R. E. *Anal. Biochem.* **1978**, *84*, 116–126.

Marjorie B. Medina*[1]
John G. Phillips[2]

[1]Eastern Regional Research Center
Agricultural Research Service
U.S. Department of Agriculture
Philadelphia, Pennsylvania 19118
[2]Consulting Statistician
Northeastern Region
Agricultural Research Service
U.S. Department of Agriculture
Philadelphia, Pennsylvania